Genotyping and phenotyping epilepsies of childhood

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Chapter 1

Introduction

I am about to discuss the disease called "sacred." It is not, in my opinion, any more divine or more sacred than any other diseases, but has a natural cause…

Its origin, like that of other diseases, lies in heredity...
The fact is that the cause of this affection is... the brain...
My own view is that those who first attributed a sacred character to this malady were like the magicians, purifiers, charlatans, and quacks of our own day... So that there is no need to put the disease in a special class and to consider it more divine than the others...
Each has a nature and a power of its own; none is hopeless or incapable of treatment.[1]

Hippocrates, 460–370 BC, on epilepsy

Hippocrates was revolutionary for his time in arguing that the origin of epilepsy lies in 'heredity'. Although hereditary does not always equal genetic, we now have robust evidence that genetic variants can cause or contribute to epilepsy. However, we still have a long way to go to fully understand the intriguing role of genetics in epilepsy.

To comprehend the current challenges in epilepsy genetics addressed in this thesis, it is helpful to learn more about epilepsy phenotyping and genotyping. Whereas epilepsy phenotyping concentrates on describing the clinical presentation, epilepsy genotyping focuses on identifying the underlying genetic cause. Integration of information on epilepsy phenotype and genotype is important in clinical practice and research settings in order to find new genes and risk factors for epilepsy, to better understand the disease and its presentation and to improve its management and outcome.

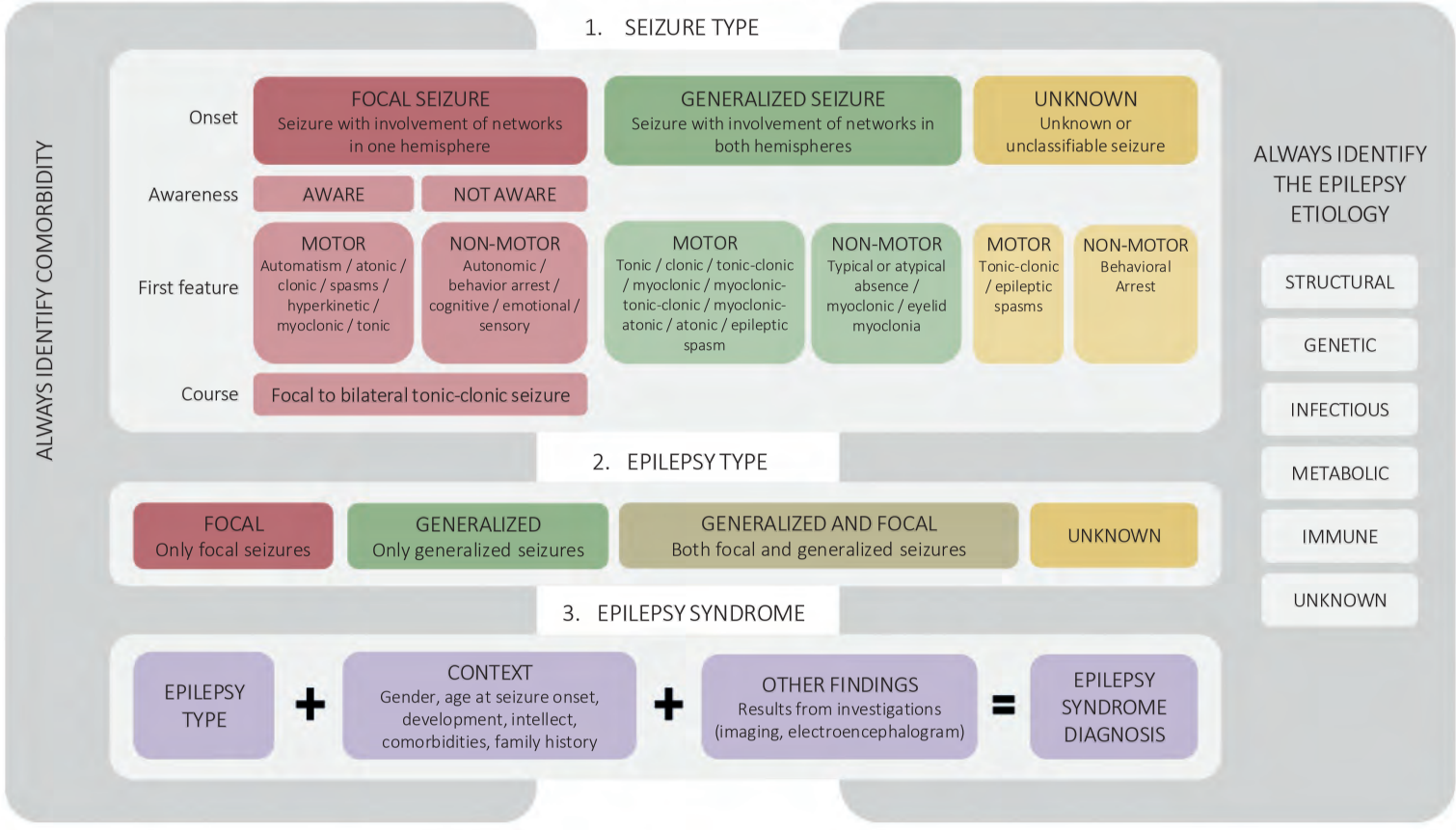
EPILEPSY PHENOTYPING

An epileptic seizure is defined as a transient occurrence of signs and/or symptoms due to abnormal excessive or synchronous neuronal activity in the brain.[2] Seizures can be provoked by acute disturbances in the body, such as hypoglycemia or acute infection, or can occur unprovoked. Patients are diagnosed with epilepsy if they present with any of the following three conditions: 1. at least two unprovoked seizures occurring >24 hours apart, 2. one unprovoked (or reflex) seizure and a probability of further seizures in the next 10 years similar to the general recurrence risk after two unprovoked seizures (at least 60%), or 3. a diagnosis of an epilepsy syndrome.[3] Epilepsy is diagnosed in 41-87 children per 100,000 children each year.[4]

Epilepsy is not a single disease entity, but should be considered as a group of disorders. Its presentation, etiology, management and prognosis can vary significantly for the different epilepsies. Epilepsy therefore warrants further classification for clinical and research purposes. The International League Against Epilepsy (ILAE) has made several classification guidelines.[5–11] In the most recent guideline from 2017, they recommended classifying epilepsy at three consecutive levels: 1. seizure type, 2. epilepsy type, and 3. epilepsy syndrome.[12] The flowchart in Figure 1 demonstrates how to classify epilepsies using this three-step method. Depending on the availability of clinical information, epilepsy can be classified up to level 1, 2 or 3.

1. **Seizure type.** Epileptic seizures should be classified based on their onset as focal, generalized or unknown.[12,13] Epilepsy is a disorder involving neuronal networks and all seizures are hypothesized to occur somewhere within a network.[14,15] Focal seizures are limited to networks within one hemisphere, while generalized seizures rapidly engage bilaterally distributed networks.[16] Both clinical and electroencephalogram (EEG) data can help to distinguish focal and generalized seizures. If more detailed clinical information on the seizures is available, they can be further classified based on the level of awareness (for focal seizures only) and by describing their first feature.[12,13] Focal seizures can evolve to bilateral tonic-clonic seizures (previously called 'secondary generalized seizures').[12,13,17]
2. **Epilepsy type.** Since individuals can have both focal and generalized seizures, a second level of classification has been introduced: epilepsy type. Epilepsy type can be focal, generalized or combined generalized and focal.[12]
3. **Epilepsy syndrome.** To diagnose the epilepsy syndrome, one should also consider the setting in which seizures occur. This setting includes the patient's gender, age at seizure onset, developmental course, family history, and results from additional investigations such as EEG and imaging. Recognition of a distinctive clinical and EEG pattern allows a syndrome diagnosis.[12]

Figure 1: Flowchart for the classification of epilepsies following a three step method. Modified after Fisher et al. 2017 & Scheffer et al. 2017. [12,13]



Comorbidities

In parallel with classifying the epilepsy from seizure type to epilepsy syndrome, it is important to identify comorbidities. Examples of comorbidities include intellectual disability, movement disorders, autism spectrum disorders and other psychiatric and behavioral disorders. Pro-active screening for the presence of comorbidities is vital because it not only helps in epilepsy syndrome and etiology diagnosis, it also enables appropriate management of comorbidities that can improve quality of life of patients and their families.

Etiology

During the whole process of phenotyping epilepsies, it is also essential to try to identify the etiology. Understanding etiology can prevent unnecessary further diagnostic investigations and may lead to better counseling, management and, possibly, outcome. Six epilepsy etiology groups have been proposed: genetic, structural, infectious, metabolic, immune and unknown.[12] For some epilepsies, etiology can be classified into more than one etiology category. For example, pyridoxine-dependent epilepsy due to a pathogenic *ALDH7A1* variant has a metabolic and genetic etiology while *DEPDC5*-gene-related epilepsy with a cortical malformation has a genetic and structural etiology. For the genetic etiology, epilepsies can be considered genetic based on the presenting phenotype or family history even when the variant itself has not yet been identified.[12]

Developmental and epileptic encephalopathy

In some epilepsies, "the epileptic activity itself may contribute to severe cognitive and behavioral impairments that is beyond what might be expected from the underlying pathology alone"[11], defined as an epileptic encephalopathy (EE). There are many different EEs that can occur in infancy and childhood, and these are often genetic.[18] However, developmental slowing and regression often precede seizure onset and cognitive deficits can persist after seizure remission. Therefore, the term *developmental and epileptic encephalopathy* (DEE) was introduced to describe the encephalopathies where the underlying genetic condition and the seizure activity together lead to developmental plateauing or regression.[12] An example of a DEE is *SYNGAP1*-encephalopathy, which is studied in this thesis.

EPILEPSY GENOTYPING

Although the role of genetics in epilepsies has been appreciated since antiquity, research on this role only started the last century and the growth in knowledge since then has been revolutionary. What follows is a historical overview of the different consecutive study types that have led to our current understanding of the complex role of genetics in epilepsies.

Family studies

The first evidence for epilepsy as a hereditary disease came from clinical family studies performed in the end of the last century. Family aggregation studies showed a 3-7 times higher incidence of epilepsy in siblings and offspring of patients with epilepsy than expected by chance.[19–21] Of patients with epilepsy, 5-21% have at least one first-degree relative sharing a diagnosis of epilepsy.[22–25] Furthermore, twin studies revealed a higher concordance for epilepsy among monozygotic versus dizygotic twins.[26–28] These studies together indicated that epilepsy has a genetic basis, but its nature and extent are unknown. Nowadays, family studies are mainly performed to evaluate the phenotypic spectrum of familial epilepsy syndromes, e.g. genetic epilepsy with febrile seizures plus[29], or to identify new familial syndromes, e.g. familial posterior quadrant epilepsies.[30]

Searching for epilepsy candidate genes

Following these family studies, different approaches were used to identify the epilepsy candidate genes and their position within the human genome (called locus). Two important study types, linkage analysis and association studies, have been performed since the eighties and nineties of the last century, respectively.

Linkage studies.

Linkage analysis can be used in families to evaluate the segregation of epilepsy with genetic markers with a known position in the genome. This technique is based on the principle that genes that reside closely together are linked during meiosis. By identifying the genetic marker that is carried by affected relatives but not by non-affected relatives, the linked gene or locus for epilepsy can be identified. The first locus for epilepsy (juvenile myoclonus epilepsy on chromosome 6) was identified in 1989, and this was followed by the discovery of many other loci for this and other epilepsy syndromes.[31,32] Linkage analysis is still used to identify new loci for epilepsy.[32]

Association studies.

Association studies aim at identifying alleles that increase the risk for developing epilepsy, called 'susceptibility alleles'. In these studies, the statistical association between a specific allele and an epilepsy syndrome is calculated by comparing the presence of this allele in large numbers of patients versus controls. Alleles found significantly more often in patients are considered susceptibility alleles. The many association studies performed to date have consistently identified a few susceptibility loci for different generalized epilepsies (1q43, 2p16.1, 2q22.3 and 17q21.32), focal and generalized epilepsy and febrile seizures (2q24.3 including *SCN1A*), febrile seizures (2q24.3, 11p14.2 and 12q21.33) or focal and generalized epilepsy (4p15.1).[33] Focusing on epilepsy treatment, an *HLA-B*1502* allele has been associated with the occurrence of carbamazepine-induced Stevens-Johnson syndrome.[34] Unfortunately, many other findings from association studies could not be replicated, mainly due to low statistical power. Future collaboration between epilepsy research groups is necessary to increase the number of participants and thereby increase the statistical power to identify new loci or genes for epilepsy or drug resistance or efficacy.[33,35,36]

Molecular genetic studies

Advances in molecular genetic techniques that disentangle the human genome in greater detail together with more adequate epilepsy phenotyping have resulted in the identification of many new genes for epilepsy. Table 1 gives an overview of the different molecular techniques that are currently available to detect genetic variants at all levels of the human genome. Here, we will discuss the three most important techniques in terms of identifying new genes and loci for epilepsy.

Sanger sequencing.

In 1995, the first disease-causing gene variant for epilepsy (previously called a mutation) was identified using Sanger sequencing in a locus identified by linkage analysis. A missense mutation in the CHolinergic Receptor Nicotinic Alpha 4 Subunit gene (*CHRNA4*) was found in a large Australian family with autosomal dominant nocturnal frontal lobe epilepsy.[37] Sanger sequencing, invented in 1977, has dominated genetic diagnostics for decades.[38] Many disease-causing variants in other genes (*SCN1B39, KCNQ240, KCNQ341, SLC2A142, SCN1A43, GABRA144*, and *GABRG245*) for other dominantly inherited epilepsies have been found following identification of their loci using linkage analysis. Currently, Sanger sequencing is mainly used to sequence a single gene selected based on a family history with variants in this gene or on an urgent need for specific treatment (such as *SCL2A1* for glut1 deficiency requiring treatment with a ketogenic diet).

Microarray.

Microarray analysis has been a widely available technique since the early 2000's. Microarray analysis detects losses (deletions) or gains (duplications) of relatively small parts of the chromosomes.[46] These deletions and duplications are called *copy number variants* (CNVs). There are two types of CNVs that can underlie epilepsy: *recurrent* and *non-recurrent* CNVs.

Recurrent CNVs occur at specific sites of the genome due to the recombination of DNA between highly homologous chromosomal regions (*non-allelic homologous recombination*). During meiosis, these highly homologous regions can misalign and an unequal crossover can occur, resulting in deletions or duplications of the DNA. These deletions and duplications can include single or multiple genes.[47] Recurrent CNVs are associated with different syndromes. For example, a 15q11.2-q13 microdeletion is associated with Angelman and Prader-Willi syndrome (OMIM 105830 and 176270, respectively) and a 22q13.33 microdeletion with Phelan-McDermid Syndrome (OMIM 606232). Epilepsy might occur in the context of these microdeletion or microduplication syndromes.

Non-recurrent CNVs are unique for every individual. These CNVs occur due to unequal crossover of DNA during meiotic (or less frequently mitotic) DNA repair processes with inconsistent breakpoints throughout the genome in regions with limited homology.[47] Identifying a *critical region of overlap* (CRO) between such non-recurrent CNVs of patients with similar phenotypes can elucidate new genes for epilepsy. For example, *GRIN2A* was identified as a candidate gene for epilepsy based on the identification of a CRO betewen 16p13.2 deletions in three patients with similar phenotypes.[48]

Subsequently, disease-causing variants in *GRIN2A* were identified in patients with epilepsy within the epilepsy-aphasia-spectrum.[49–51]

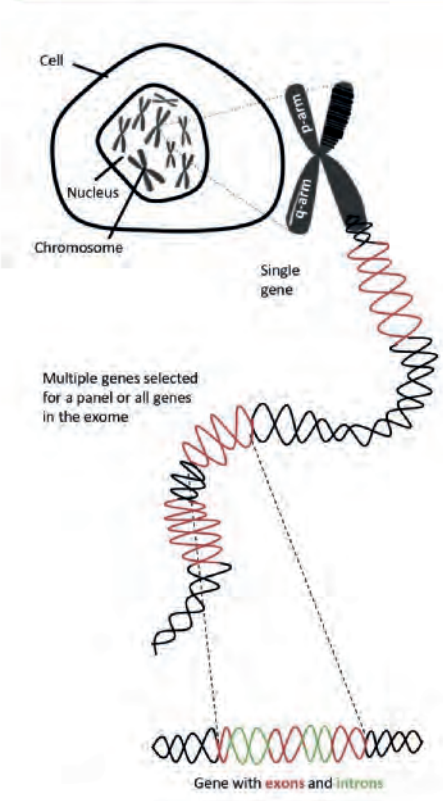
Several studies have shown that causal CNVs are present in ~5% of patients from different cohorts with generalized epilepsy, focal epilepsy or epileptic encephalopathy.[52–55] In addition, a few recurrent CNVs have been found significantly more often in patients with epilepsy compared to controls, and these are now referred to as *susceptibility CNVs* because they can increase the risk of developing epilepsy. Deletions on chromosomes 1q21.1, 15q11.2, 15q13.3, 16p11.2 and 16p13.11 and duplications on chromosome 16p11.2 are well-known examples of susceptibility CNVs.[52,55–57]

Next generation sequencing.
With the introduction of Next Generation Sequencing (NGS) in 2008-2009, we entered a new, very exciting era in epilepsy genetics research and diagnostics. NGS enables sequencing of multiple genes, of the whole exome or of the whole genome in a single test run. This has enabled two major advances. First, variants can be identified in genes that would otherwise not have been selected for genetic testing. NGS may therefore identify many novel candidate genes for epilepsy and, indeed, the list of epilepsy candidate genes has grown tremendously since its introduction.[58–60] Second, gene-related phenotypic spectra can be expanded when gene variants are found in patients with phenotypes that were not yet associated with this gene. For example, *SYNGAP1* was identified as a gene for an epileptic encephalopathy after it was first identified as a gene for intellectual disability.[61,62]

GENETIC DATA INTERPRETATION

The genome of each individual, affected or non-affected, differs at 4-5 million sites from the reference human genome and harbors 2,100 to 2,500 structural genetic variants.[63] Not surprisingly, the genome-wide genetic techniques discussed above also identify numerous variants that are not associated with disease. Therefore, our challenge is to interpret these variants and pinpoint the disease-causing variant in our patients. International guidelines have been established to classify variants based on their pathogenicity as benign, likely benign, of unknown significance, likely pathogenic or definitely pathogenic.[64] Variants are first filtered based on their prevalence in national and international population databases such as the genome of the Netherlands (GoNL)[65], genome Aggregation Database (gnomAD)[66], 1000 Genomes[63], dbSNP[67] and the database for genomic variants (DGV)[68]. A variants is considered likely benign if it is present in more than 1% of the population.[64] For the variants that are not considered likely benign, the variant's inheritance, location, in silico predicted or functionally tested effects, presence in other patients with similar phenotypes are evaluated to determine its pathogenicity.[64] With the introduction of whole exome sequencing, and possibly whole genome sequencing in the near future, the interpretation of genetic testing is becoming more difficult and time-consuming as ever more variants are being identified.

Table 1: Genetic techniques and their indications, advantages, disadvantages

Human genome level for detecting variants	Test	Variations detected	Typical indication	Advantages	Disadvantages
	Karyotyping	Large chromosomal rearrangements	Suspicion of a chromosomal disorder	- Able to detect ring chromosomes and balanced rearrangements	- Low resolution coverage of genome - Possibility of incidental findings for diseases other than epilepsy
	Microarray	CNVs, regions of homozygosity, uniparental isodisomy	Epilepsy "plus" (developmental delay, behavioral problems or dysmorphisms)	- High resolution coverage of genome	- Misses balanced chromosomal rearrangements - Possibility of incidental findings for diseases other than epilepsy
	Sanger sequencing	Sequence variants in single or up to a few genes	Epilepsy syndrome known to be caused by a single gene variant	- No possibility of incidental findings due to careful selection of genes	- Pre-selection of gene based on clinical presentation is necessary - Time- and cost-expensive to sequence genes individually - Not possible to detect intragenic CNVs
	Epilepsy gene panel	Sequence variants within a subset of genes	Genetic epilepsy	- Low possibility of incidental findings due to careful selection of genes	- Gene panels need to be updated frequently - Some laboratories: not possible to detect intragenic CNVs
	Whole exome sequencing with epilepsy filter	Sequence variants in the exomes, but only analyzes of subset of genes	Genetic epilepsy	- Low possibility of incidental findings due to careful selection of genes, which can be extended easily	- Some laboratories: not possible to detect intragenic CNVs
	Whole exome sequencing	Sequence variants within in the exomes	Genetic epilepsy without a gene variant identified using a gene panel	- No need for selection of genes if no clinical clue	
	Whole genome sequencing	Sequence variants and CNVs in the whole genome (introns + exons)	Currently only applied in research settings	- No need for selection of genes if no clinical clue - Data available to detect intronic variants and exonic copy number variants	- Not yet possible to reliably interpret the relevance of intronic variants - Possibility of incidental findings for diseases other than epilepsy

CONCEPTUALIZING EPILEPSY GENETICS

Over the last few decades our understanding of the genetic basis of epilepsies has increased tremendously and this knowledge can be summarized in three important concepts.

Epilepsy gene products are part of different pathways

When the first ion-channel-coding genes for epilepsies were discovered, the idea was raised that epilepsy could be channelopathy.[60] This concept has however been overturned by the discovery in 2002 of *LGI1*, a non-ion-channel-coding gene for autosomal dominant lateral temporal lobe epilepsy.[69] Following the identification of many non-ion-coding-channel genes for epilepsy, we realized that epilepsy gene products can be part of different pathways and thereby affect neuronal function differently. These pathways include DNA repair, transcriptional regulation, axon myelination, metabolite and ion transport, peroxisomal function, cell-cell adhesion, neurite formation, interneuron migration, apoptosis and blood-brain barrier transport.[70] Notably, gene products within the same pathway can often result in a similar epilepsy phenotype. The identification of *DEPDC5* as the causal gene for focal epilepsy and cortical brain malformations in the mammalian target of rapamycin (mTOR) pathway, known to be involved in tuberous sclerosis, nicely illustrates this.[71,72] Even more striking, sequencing of other genes in the mTOR pathway identified variants in *NPRL2* and *NPRL3*, both associated with a similar phenotype.[73]

Epilepsy encompasses different modes of inheritance

We have learned that epilepsy can be inherited following different modes of inheritance. Although some epilepsies are caused by a single gene variant (*monogenic inheritance*), the majority of epilepsies follow a complex inheritance where many genes (*polygenic inheritance*) or a combination of genes and environmental factors (*multifactorial inheritance*) increase the risk of developing epilepsy.[74] In monogenic epilepsies, the disease-causing gene variant does not necessarily lead to disease in all relatives carrying this variant, a phenomenon called *incomplete penetrance*. Alternatively, a relative might be affected yet not carry the disease-causing variant, a condition referred to as a *phenocopy*.

Phenotypic and genetic heterogeneity play a key role in epilepsy

Phenotypic heterogeneity (also called *pleiotropy*) refers to a single genetic disorder leading to different epilepsy syndromes: e.g. mutations in KCNQ2 can cause benign neonatal and infantile epilepsy as well as severe neonatal epileptic encephalopathy.[75,76] *Genetic heterogeneity* means that a single epilepsy syndrome can be caused by pathogenic variants in different genes. For example, benign infantile epilepsy can by caused by variants in *KCNQ2*, *KCNQ3*, *SCN2A* or *PRRT2*.[75] For some genes, such as *SCN1A*, clear genotype-phenotype correlations have been identified that explain the *SCN1A* phenotypic heterogeneity by focusing on the mutation type and location of SCN1A variants in relation to the phenotype.[77] For other genes, such as *SYNGAP1* and *GRIN2A*, we still lack a clear genotype-phenotype correlation.[78,79]

ADVANCES AND CHALLENGES IN EPILEPSY GENETICS IN CURRENT CLINICAL PRACTICE

The yield of genetic testing

Not long ago it was unimaginable that we would now know of 700 genes related to epilepsy.[58] Even so, we still cannot identify the genetic cause in a significant number of epilepsy cases that are suspected to be genetic. Only one large and two very small studies addressed the yield of microarray in clinical hospital cohorts of patients with all types of epilepsies: the yield varied between 9% and 40%.[55,80,81] In one of these studies, new loci of interest for epilepsy were identified.[55] In **chapter 2**, we describe the diagnostic yield of microarray in a large clinical hospital cohort of children with epilepsy in the Netherlands and the possible novel CNVs for epilepsy.

With our increasing ability to identify the underlying genetic causes of epilepsy, genetic testing is offered more and more in clinical practice. Using these techniques, we hope not only to improve epilepsy care but also to provide answers to longstanding questions from patients and parents about the epilepsy cause and prognosis. However, the impact on patients and their parents of genetic counseling and receiving a genetic diagnosis has not yet been studied.[82,83] In **chapter 3**, we present the results of a study evaluating empowerment and anxiety during a genetic counseling trajectory in patients with epilepsy or their parents in whom genetic causes were and were not identified.

Genotype-phenotype studies

With the introduction of NGS, many new genes for epilepsy have been identified in different cohorts of patients selected based on their phenotype. Due to this ascertainment, the clinical features associated with these genes is often biased in the original papers. To unravel the full gene-related phenotypic spectrum and the genotype-phenotype correlations, patients should be selected based on their genotype and subsequently be phenotyped (a process called *reverse phenotyping*). In **chapter 4** and **chapter 5**, we report the phenotypic spectra and genotype-phenotype correlations for *SYNGAP1* and *GRIN2A*, respectively. In **chapter 6**, we show that psychotic disorders are a new, later-onset manifestation of *PCDH19* Girls Clustering Epilepsy. **Chapter 7** describes a case report of a boy with a deletion of the *STX1B* gene and a new phenotype: epilepsy with myoclonic-atonic seizures (previously called myoclonic astatic epilepsy).

In **Chapter 8**, we evaluate the presence of *PRRT2*-related phenotypes in patients with 16p11.2 microdeletions including PRRT2 and the 16p11.2 microdeletion syndrome.

All efforts to identify genes for epilepsy are driven by the hope of improving epilepsy care. For now, however, the identification of new epilepsy genes has been translated into better management of seizures in only a few cases. Currently, 'personalized' medicine for epilepsy has been established in only two scenarios: a ketogenic diet for patients with SLC2A1 variants and treatment with pyridoxine for patients with *ALDH7A1 variants*.[84] In **chapter 9**, we report the efficacy of carbamazepine as a

sodium-channel blocker in the case of a girl with infantile epilepsy due to a *SCN2A* sequence variant and. This case report is an example of precision medicine.

In **chapter 10** we discuss our results in a general perspective and we propose a diagnostic algorithm for genetic testing for epilepsy in clinical practice.

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PART I

The yield of genetic testing